CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem. Funct. **18**, 99–102 (2000)

Erythrocyte Lipid Peroxidation and Antioxidants in Cigarette Smokers

UMA CODANDABANY*

Department of Biochemistry, Dr A.L. Mudaliar Post Graduate Institute of Basic Medical Sciences, Taramani Madras 600113, Tamil Nadu, India

The present study has analysed the relationship between lipid peroxidation and antioxidant status in erythrocytes from 30 adult male cigarette smokers and an equal number of age and sex-matched normal subjects. Erythrocyte lipid peroxidation was markedly increased. The enzymic antioxidants were decreased in erythrocytes of cigarette smokers. The present study highlights the occurrence of lipid peroxidation and possible breakdown of antioxidant status in cigarette smoking. Copyright © 2000 John Wiley & Sons, Ltd.

KEY WORDS — smoking; lipid peroxidation; antioxidants; erythrocytes

INTRODUCTION

Free radical-mediated processes have been implicated in tobacco carcinogenesis.[1] Active oxygen radicals are generated indirectly in pulmonary alveolar macrophages stimulated by cigarette smoke, which therefore becomes an important tumour promoter besides manifesting its carcinogenic action.[2] Lipid peroxidation causes considerable alteration in the functional and structural organization of the cell membrane.[3] The smoking-related inflammatory response may stem from an imbalance between oxidant and antioxidant status.[4] The erythrocyte membrane is particularly susceptible to oxidative damage due to a high content of iron, polyunsaturated fatty acids and its role as an oxygen transporter.[5]

The formation of lipid peroxidation products is normally prevented or scavenged by a host of antioxidants. The normal erythrocyte is highly resistant to free radical damage due to its multilevel antioxidant defence mechanisms.[6] Superoxide radicals are cytotoxic. Their efficient clearance by superoxide dismutase (SOD) creates hydrogen peroxide which is considerably less toxic and which is further reduced by catalase to water. They (SOD and cataase) prevent formation of the extremely toxic hydroxyl radical.[7] Glutathione, an important cellular reductant is involved in protection against free radicals, peroxides and other toxic compounds.[8] Erythrocyte glutathione peroxidase is responsible for the detoxification of peroxide.[9] The ability of the antioxidants to destroy free radicals protects both the structural integrity of cells and tissues[10] and also against the deleterious effects of lipid peroxidation.[11]

Thirty per cent of the total cancer mortality in men and women is associated with smoking according to present estimates in the USA.[12] There is evidence that cigarette smoke is a major risk factor for many types of human cancer[13] such as oral and pharyngeal cancer because it contains oxidant species.[14] However, the involvement of free radicals in cigarette smoking has not been firmly established. The erythrocyte is an excellent model for studies on lipid peroxidation and antioxidant status due to its simplicity, easy availability and ease of isolation. The present study was therefore undertaken to assess the extent of lipid peroxidation and the status of antioxidants in erythrocytes of cigarette smokers.

MATERIALS AND METHODS

Blood samples were obtained by puncture of an arm vein and were collected into heparinized tubes. Plasma was separated from packed cells and cell counting was done as described.[15] Cells were plated

* Correspondence to: Dr Uma Codandabany, 39, bd. Clemenceau, 67000 Strasbourg, France. Tel: 0033 388356143.

Received 27 April 1999
Accepted 18 August 1999

in 30-mm Petri dishes at a density of $1{\cdot}25 \times 10^5$ cells per dish and allowed to grow. Cells were serum deprived for 48 h followed by effector treatment. Cells were rinsed extensively and counted after tryptan blue staining. The erythrocyte membrane was prepared by the method of Dodje *et al.*[16] as modified by Shanmugasundaram *et al.*[17] followed by haemoglobin measurement as described by Drabkin and Austin[18] and the content of the erythrocyte membrane protein measured according to the method of Lowry *et al.*[19]

Enzyme assays

Superoxide dismutase was assayed as described by Misra and Fridovich.[20] The hemolysate was diluted with water, ethanol and choloroform. To the supernatent, 0·1 M carbonate–bicarbonate buffer pH 10·2 was added. The reaction was initiated by the addition of 3 mM epinephrine and the change in absorbance per minute measured at 50 per cent inhibition of adrenochrome transition enzyme was taken as one enzyme unit. The procedures used for catalase and glutathione peroxidase were similar to those used by Sinha[21] and Rotruck *et al.*[22] respectively. Catalase activity was determined by measuring the decomposition of hydrogen peroxide at 570 nm. Glutathione peroxidase was assayed by the addition of incubation mixture (containing phosphate buffer 0·32 M (pH 7·0), 10 mM sodium azide, 4 mM reduced glutathione, 2·5 mM hydrogen peroxide) to the haemolysate and incubation at 37°C. The reaction was terminated by addition of 10 per cent trichloroacetic acid followed by the addition of 5·5′-dithiobis (2-nitro benzoic acid) in 1 per cent sodium citrate (DTNB). Colour development was read at 412 nm. The reduced glutathione was measured by the method of Beutler and Kelly.[23] To an aliquot of lysate was added 10 per cent trichloroacetic acid. To the filtrate obtained, was added 0·3 M disodium hydrogen phosphate and DTNB reagent as prepared previously. The absorbance of the colour that developed was measured at 412 nm.

Lipid peroxidation determination

The level of lipid peroxidation was assessed by the method of Cyanoman *et al.*[24] To the membrane suspension was added 0·1 M phosphate buffer pH 7·4 and 40 per cent trichloroacetic acid. To the supernatent 1 per cent thiobarbituric acid was added and the contents were boiled for 20 min, cooled and read at 535 nm.

Statistical analysis was carried out using the Student's *t*-test.

RESULTS AND DISCUSSION

The investigation was designed to determine the deleterious effects due to oxidative stress produced by cigarette smoking. Table 1 shows the haematological record of the subjects investigated. Fasting blood glucose in smokers is slightly higher than non-smokers, although the changes are statistically significant. Colbert *et al.*[25] concluded that long-term smoking independent of acute smoking, increases the dependence on blood glucose as a fuel during rest and sustained submaximal exercise. Haemoglobin decreased from 14·3 to 12·1 g dl^{-1} and the cell count from 5·7 to $4{\cdot}1 \times 10^6$ μl^{-1} compared to control subjects. It is clear from the data that as the cell count per unit volume decreases the haemoglobin is bound to fall also.

Table 2 shows the extent of lipid peroxidation in the erythrocyte membrane of normal subjects and smokers. Lipid peroxidation was significantly increased in smokers to about 319 µmole of MDA liberated per 10^{12} cells compared with approximately 273 µmole of MDA liberated per 10^{12} cells in normal subjects. Lipid peroxidation has been implicated in a number of deleterious effects such as increased membrane rigidity, osmotic fragility, decreased cellular deformability, reduced erythrocyte survival and lipid fluidity.[26] Red blood cells of smokers were more fragile than those from control subjects. This can be attributed to lipid peroxidation of the red cell membrane. Oxidative stress has

Table 1. Haematology of the subjects investigated.

Parameters	Non-smokers	Smokers
Glucose (mg dl^{-1})	$89{\cdot}15 \pm 7{\cdot}13$	$95{\cdot}22 \pm 4{\cdot}30$*
Haemoglobin (g dl^{-1})	$14{\cdot}65 \pm 1{\cdot}30$	$12{\cdot}17 \pm 0{\cdot}85$†
Red blood cell count (10^6 μl^{-1})	$5{\cdot}73 \pm 0{\cdot}69$	$4{\cdot}06 \pm 0{\cdot}51$†

The results represent the mean ± SD from 30 subjects in each group. * $p < 0{\cdot}001$; † $p < 0{\cdot}01$.

Table 2. Level of lipid peroxidation in erythrocyte membranes of non-smokers and smokers.

Parameters	Non-smokers	Smokers
Lipid peroxidation (µmole of MDA liberated per 10^{12} cells)	$273{\cdot}06 \pm 9{\cdot}24$	$319{\cdot}20 \pm 8{\cdot}31$*

The results represent the mean ± SD from 30 subjects in each group. * $p < 0{\cdot}001$.

Table 3. The antioxidant status in erythrocytes and erythrocyte membranes in non-smokers and smokers.

Parameters	Non-smokers	Smokers
Erythrocytes		
Superoxide dismutase (Units mg^{-1} Hb)	$3{\cdot}52 \pm 1{\cdot}03$	$2{\cdot}77 \pm 0{\cdot}18$*
Glutathione peroxidase (μg of reduced glutathione liberated mg^{-1} Hb min^{-1})	$5{\cdot}38 \pm 0{\cdot}04$	$4{\cdot}85 \pm 0{\cdot}03$*
Reduced glutathione (μg mg^{-1} Hb)	$0{\cdot}181 \pm 0{\cdot}005$	$0{\cdot}166 \pm 0{\cdot}004$*
Erythrocyte membrane		
Catalase (μmole of H_2O_2 consumed 10^{-12} cells min^{-1})	$4{\cdot}40 \pm 0{\cdot}04$	$3{\cdot}40 \pm 0{\cdot}06$*

The results represent the mean ± SD from 30 subjects in each group. * $p < 0{\cdot}01$.

been reported to reduce the whole cell deformability. Malondialdehyde cross-linking and lipid peroxidation have been suggested to play a role in the immunological destruction of erythrocytes.[27] The decrease in erythrocyte count in smokers may be a consequence of enhanced lipid peroxidation of erythrocytes.

The antioxidant status in erythrocytes (superoxide dismutase, glutathione peroxidase and glutathione reductase) and in the erythrocyte membrane (catalase) of normal subjects and smokers is presented in Table 3. All the antioxidant enzymes were significantly decreased in smokers compared with non-smoking individuals. The antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase, are reduced by 20–30 per cent, while glutathione reductase is reduced by less than 10 per cent in the blood samples from smokers (Table 3).

Decreased activity of superoxide dismutase has been reported in pathological conditions.[28,29] Superoxide, a highly diffusible radical which can transverse membranes, causing deleterious effects, and active oxygen species are generated by the active metabolites of naphthylamines during inhalation and absorption of cigarette smoke by pulmonary cells.[30] It is possible that increased lipid peroxidation in smokers and low activity of superoxide dismutase within red cells are due to active oxygen radicals produced by the cigarette smoke.

The antioxidant enzyme catalase is widely distributed in all animal tissues and high activity is found in red blood cells. Several studies have shown a decrease in catalase activity in cancer as part of a severe impairment of antioxidant systems.[31,32] Catalase has been suggested to play an important role in the protection of the erythrocyte against oxidative stress.[33] Studies have shown that the administration of catalase results in protection against hydrogen peroxide-mediated lipid peroxidation.[31–34] The increase in lipid peroxidation levels in smokers can be correlated with the reduction in catalase activity.

Glutathione is a metabolic regulator and a putative indicator of health. Blood glutathionne levels are believed to be predictors of morbidity and mortality.[35] Low glutathione levels and the decreased activity of glutathione peroxidase found in smokers in the present study supports the hypothesis that smoking leads to a greater oxidative burden and depletion of antioxidant defences. The decrease in red cell glutathione peroxidase activities in smokers may be due to increased scavenging of lipid peroxides. Red blood cell glutathione and glutathione peroxidase have a major role in protecting the erythrocyte membrane against oxidation.[36] A decreased content of red blood cell glutathione in smokers may be one of the main factors responsible for the increased fragility of erythrocytes.

The present findings indicate that erythrocytes from smokers are more susceptible to lipid peroxidation as a consequence of insufficient antioxidant potential and greater oxidative burden. The low antioxidant status of smokers may predispose them to oxidant- and cytokine-inflicted tissue damage and disease, which may manifest itself as coronary heart disease, atherosclerosis and cancer.

ACKNOWLEDGEMENTS

The author is grateful to Dr. K. Radha Shanmugasundaram, Professor and Head of the Department of Biochemistry, Madras University, for her guidance and constant encouragement.

REFERENCES

1. Pryor, W. A. (1982). Free radical biology xenobiotics. Cancer and ageing. *Ann. NY Acad. Sci.*, **393**, 1–22.
2. Kensler, T. W., Bush, D. M. and Kozumbo, W. J. (1983). Inhibition of tumour promotion by a biomimetic superoxide dismutase. *Science*, **221**, 75–77.

3. Borochov, H., Abbott, E. R., Schachtr, D. and Shinitzky, M. (1979). Modulation of erythrocyte membrane proteins by membrane cholesterol and lipid fluidity. *Biochemistry*, **18**, 251–255.
4. Shaper, A. G., Pocock, S. J., Walker, M., Phillips, A. N., Whitehead, T. P. and Macsarlane, P. W. (1985). Risk factors for ischaemic heart disease. The prospective phase of the British regional heart study. *J. Epidemiol.*, **39**, 197–207.
5. Hebbel, R. P. (1986). Erythrocyte antioxidants and membrane vulnerability. *J. Lab. Clin. Med.*, **107**, 401–405.
6. Burton, G. W., Joyce, A. and Ingold, K. V. (1983). Is vitamin E the only lipid-soluble chain breaking antioxidant in human blood plasma and erythrocyte membranes? *Arch. Biochem. Biophys.*, **221**, 281–285.
7. Brown, K. and Fridovich, I. (1983). Superoxide radical and superoxide dismutase. Threat and defence. *Acta Physiol. Scand.*, **494**(Suppl.), 9–18.
8. Vina, R. J., Saez, S. T. and Vina, J. (1989). The physiological functions of glutathione. In: *Handbook of Free Radicals and Antioxidants in Biomedicine* (Miquel, J., Quintanilla, A. T. and Weber, H., eds). CRC Press, Inc: Boca Raton, pp. 121–132.
9. Mills, G. C. (1957). Haemoglobin catabolism. Glutathione peroxidase, an erythrocyte enzyme which protects haemoglobin from oxidative breakdown. *J. Pharmacol. Toxicol.*, **43**, 291–298.
10. Bendich, A. (1993). Physiological role of antioxidants in the immune system. *J. Dairy Sci.*, **76**, 2789–2794.
11. Jernstorm, B., Morgenstern, R. and Moldeus, P. (1993). Protective role of glutathione, thiols and analogues in mutagenesis and carcinogens. In: *Antimutagenesis and Anticarcinogenesis Mechanism* (Bronzetti, H. H., DeFlora, S., Waters, M. D. and Shankel, D. M., eds). Plenum Press: New York, pp. 137–148.
12. Greenwald, P., Sondik, E. and Lynch, B. S. (1986). Diet and chemoprevention of NCI's research strategy to achieve national control objectives. *Ann. Rev. Pub. Health*, **64**, 73–78.
13. Hoffmann, D. and Hecht, S. S. (1990). Advances in tobacco carcinogenesis. In: *Handbook of Experimental Pharmacology*, Vol. I. Springer: Berlin, Heidelberg, New York, pp. 63–102.
14. Dilsiz, N., Olcucu, A., Cay, M., Naziroglu, M. and Cobanoglu, D. (1999). Protective effects of Senelnium, Vit. C and Vit. E against oxidative stress of cigarette smoke in rats. *Cell. Biochem. Funct.*, **17**, 1–7.
15. Krady, M. M., Freyermuth, S., Rogue, P. and Anant, N. M. (1997). Pervandate elicits proliferation and mediates activation of mitogen-activated protein (MAP) kinase in the nucleus. *FEBS Letts.* **412**, 420–424.
16. Dodje, J. F., Mitchell, G. and Hanahan, D. J. (1968). The preparation and chemical characterisation of haemoglobin-free ghosts of human red blood cells. *Arch. Biochem. Biophys.*, **100**, 119–130.
17. Shanmugasundaram, K. R., Padmavathi, C., Sujatha, A., Vidyalakshmi, N. and Vijayan, V. K. (1992). Exercise induced cholesterol depletion and Na^+, K^+-ATPase activities in human red blood cell membrane. *Exp. Phy.*, **77**, 933–936.
18. Drabkin, D. L. and Austin, J. H. (1932). Spectrophotometric studies. spectrophotometric constants for common haemoglobin derivatives in human, dog and rabbit blood. *J. Biol. Chem.*, **98**, 719–733.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with Folin's phenol reagent. *J. Biol. Chem.*, **193**, 265–273.
20. Misra, H. P. and Fridovich, I. (1972). The role of superoxide anion in the auto oxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.*, **247**, 3170–3175.
21. Sinha, K. A. (1972). Colorimetric assay of catalase. *Anal. Biochem.*, **47**, 289–294.
22. Rotruck, J. T., Poe, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D. C. and Hoekstra, W. G. (1973). Selenium: Biochemical role as a component of glutathione peroxidase. *Science*, **179**, 588–590.
23. Beutler, E. and Kelley, B. M. (1963). The effect of sodium nitrate on red cell glutathione. *Experientia*, **19**, 96–97.
24. Cyanoman, A. A., Isenberg, J. A. and Ngayen, W. H. (1985). Erythrocyte malondialdehyde release *in vitro*. A functional measure of vitamin E status. *Clin. Chim. Acta*, **151**, 169–176.
25. Colbert, S. R., Casazza, G. A., Horning, M. A. and Brooks, G. A. (1994). Increased dependence on blood glucose in smokers during rest and sustained exercise. *J. Appl. Physiol.*, **76**, 26–32.
26. Jain, S. K., Mohandas, N., Clark, M. R. and Shobet, S. B. (1983). The effect of malondialdehyde, a product of lipid peroxidation on the deformability, dehydration and ^{51}Cr survival of erythrocytes. *Br. J. Haematol.*, **53**, 247–252.
27. Hebbel, R. P. and Miller, W. J. (1984). Phagocytosis of sickle erythrocytes: Immunological and oxidative determinants of haemolytic anaemia. *Blood*, **64**, 733–735.
28. Oberley, L. W. and Buettner, G. R. (1979). Role of superoxide dismutase in cancer: a review. *Cancer Res.*, **39**, 1141–1149.
29. Deonisi, D., Galeotti, T., Terranove, T. and Azzi, A. (1975). Superoxide radicals and hydrogen peroxide formation in mitochondria from normal and neoplastic tissues. *Biochim. Biophys. Acta*, **403**, 292–300.
30. Nakayama, T., Kimura, T., Kodama, T. and Nagata, C. (1983). Generation of hydrogen peroxide and superoxide anion from active metabolites of naphthylamines and azodyes. *Gann*, **4**, 765–769.
31. Corrocher, R., Carasil, M., Bellisola, G., Gabrielli, G. B., Nicoli, N., Guidi, G. C. and Sander, G. D. (1986). Severe impairment of antioxidant systems in human hepatoma. *Cancer*, **58**, 1658–1662.
32. Kaplan, J. H. and Groses, J. N. (1972). Liver and blood cell catalase activity of tumour bearing mice. *Cancer Res.*, **26**, 1190–1194.
33. Scott, M. D., Lubin, B. H. and Lin, Zuo and Kuypers, F. A. (1991). Erythrocyte defence against hydrogen peroxide pre-eminent importance of catalase. *J. Lab. Clin. Med.*, **118**, 7–16.
34. Holbach, S. (1997). A highly active glutathione peroxidase in red blood cells of normal and acatalasemic mice. *Experientia*, **33**, 1571–1572.
35. Robinson, M. E., Godfrey, P. J., Thompson, L. D., Rea, H. M. and Van Riji, A. M. (1979). Blood selenium and glutathione peroxidase activity in normal subjects and in surgical patients with and without cancer in New Zealand. *Am. J. Clin. Nutr.*, **32**, 1477–1485.
36. Sude, R. A. and Hoekstra, W. G. (1980). Structure, synthesis and function of glutathione peroxidase. *Nutri. Res.*, **8**, 265–273.